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ANAPHYLATOXIN AND ANAPHYLAXIS*

I. TRYPANOSOME ANAPHYLATOXIN

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SUMMARY

FOREWORD

In the course of experimental work on immunity against the trypanosomes of surra and nagana it was found that severe toxic effects with marked hypothermia often followed the injection of autolyzed suspensions of the organisms. When such injections were repeated the chronic intoxication thus induced usually ended fatally, and while a certain minimal protection could be established in carefully treated animals, it was evident that the poisonous action observed was a bar to the production of a higher degree of immunity. As no advance seemed to be possible without a clear understanding of the nature of this intoxication, the trend of the investigation had to be changed into a new direction. At first the poisonous effects were identified with the 'trypanotoxin' of Laveran,[1] but the similarity to

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[1] Laveran et Pettit, Bull. Soc. path. exot., 1911, 4, pp. 42, 680.

that of the so-called anaphylatoxins was such that it led to a study of trypanosomes in their relation to the production of this poison.

Exactly similar conditions have been encountered in connection with the so-called bacterial 'endotoxins' from the time of Pfeiffer's[2] work on the cholera vibrio down to the more recent studies of Rosenow[3] on pneumococci. Indeed, the bacterial anaphylatoxins have been identified with such endotoxins. The same problem reappears in recent studies on the toxicity of blood and of organ extracts of animals, and also of plant extracts.

The results obtained in this study of trypanosomes confirmed and extended previous work, but it was not sufficient to know that trypanosomes in some way give rise to anaphylatoxin. It was desirable to learn, if possible, the exact rôle played by the organism in the production of the poison, as well as the source and nature of the latter; in short, the mechanism of the reaction as a whole called for solution. As the work progressed it became necessary to apply similar questions to other of the so-called antigens, such as bacteria, agar, peptone, organ extracts, etc.; thence, the road led to the larger problem of anaphylaxis proper and even to the phenomenon of blood coagulation. It will be seen therefore that the work has extended well beyond its original scope, with the advantage that whenever an impasse was met in one direction another lead was available. Without doubt the results obtained have a direct bearing on the problems of immunity, which must be approached with a conception of the nature of the mechanism different from that which has hitherto prevailed.

The essential value of a theory lies in the stimulation it gives to the search for facts which serve for its support or rejection. But facts have often but a relative worth since their meaning and value change with the point of view. In biologic science especially, theories are but creations of the day, their place being taken by others of the morrow. The study of anaphylaxis has been fruitful of many theories, but it will be necessary to add a new one which, while unifying observations of utmost diversity, provides at the same time a basic conception regarding the interpretation of many pathologic and physiologic processes.

The demonstration that bacteria and diverse other organisms and cells, on contact with normal sera, give rise to anaphylatoxin quite naturally led several workers to test trypanosomes in this regard.

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[2] Ztschr. f. Hyg. u. Infektionskr., 1894, 16, p. 268.

[3] Jour. Infect. Dis., 1912, 11, pp. 94, 235, 286.

Friedberger and Szymanowski[4] mentioned obtaining a poison from this source, but no details were given. Dold[5] merely referred to this statement. The first actual publication dealing with the production of anaphylatoxin by trypanosomes was that of Marcora,[6] who succeeded in obtaining one fatal anaphylactic shock in a guinea-pig by the injection of guinea-pig serum (4 c.c.) toxified by means of washed nagana trypanosomes. The mixture of trypanosomes and serum was kept at 37 C. for 2 hours, then in an icebox for from 12 to 18 hours, and afterward was centrifugated. Conforming with the prevailing theory, he believed that the poison was produced out of the trypanosomes. Mutermilch[7] reported a brief series of tests with the same organism. By keeping a mixture of T. brucei and guinea-pig serum for from 2 to 3 hours at 38 C., then icing it for 20 hours or more, he obtained a toxic serum which killed in from 2 to 3 minutes in dose of 3 c.c.

METHODS EMPLOYED

At the outset and to a very large extent during the course of this investigation, the serum of white rats, either normal or infected, was employed, and to this fortuitous circumstance was due in large measure the success which attended this work. The great advantage of the serum of the rat over that of the guinea-pig, rabbit, etc., lies in the fact that it readily yields a very active anaphylatoxin which is fatal in dose of 0.5 and even 0.25 c.c. With such an active reagent at our disposal it became possible to attack the various problems as they presented themselves. Furthermore, the rat itself, as a reagent, was found to possess marked advantages over other animals, and this fact proved most useful, especially in the study of anaphylaxis.

To avoid unnecessary repetition it will be well to state, once for all, that all tests, unless otherwise indicated, were made under strictly sterile conditions. Obviously, the most important manipulation consisted in bleeding the animal. Here, as in many other things, practice was essential to success.

Rats were bled from the heart by means of a pipet such as is shown in Fig. 1. This was readily made from a test tube (16 x 160 mm.) by drawing out the end and bending the narrowed portion, the two operations being made at the same time. A glass rod, passing through the cotton, served to defibrinate the blood. The etherized rat was attached to a suitable board, the heart was then exposed, grasped with a special oval-tipped forceps, and the tip of the pipet, previously broken off and flamed, was then inserted. While mouth suction was applied to the tube, the capillary was passed back and forth into the auricle and venae cavae until the rat was exsanguinated. The tip of the pipet was then sealed in the flame. In special cases in which speed was necessary, the tip was closed with sealing wax or vaselin. The blood was then defibrinated for a few minutes, after which it was transferred with a bulb pipet or poured directly into a pooling flask, or centrifuge tube, previous flaming, of course, being resorted to. The actual bleeding time was from 1 to 1½ minutes, while the entire operation did not necessarily take over 5 minutes. The duration and mode of defibrination have an important bearing on the result; it should be as short as possible.

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[4] Ztschr. f. Immunitätsf., 1911, 9, p. 379.

[5] Das Bakterienanaphylatoxin, etc., 1912.

[6] Ztschr. f. Immunitätsf., 1911, 12, p. 595.

[7] Compt. rend. Soc. de biol., 1912, 73, p. 56. Ann. de l'Inst. Pasteur, 1913, 27, p. 87.

Guinea-pigs were also bled from the heart; the heart pipet in this case being made from a larger test tube (25 x 200 mm.). The manipulations otherwise were the same as given in the foregoing. When only a small amount of blood was desired, this was often obtained with a syringe, by direct heart-puncture, through the thoracic wall. In other cases, in which speed was essential, the blood was drawn from the exposed heart into a syringe.

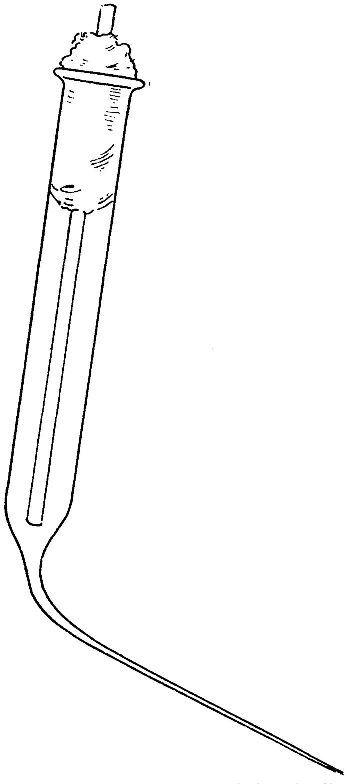

Fig. 1. The Novy heart pipet used in bleeding rats, etc.

Rabbits were usually bled from the carotid by means of large pipets similar to that described. These were made by attaching a 10-mm. glass tube to the bottom of a large test tube (30 to 45 mm. in diameter by 200 mm.). The tube was then drawn and bent at a suitable angle, sealed, and provided with a cotton plug through which a glass defibrinating rod was passed. Just before use the tip of the sterile pipet was cut off and flamed, after which it was inserted into the exposed carotid. Direct heart-puncture by means of a syringe was resorted to when only a small amount of blood was desired. The Latapie holder was invaluable in work with rabbits and guinea-pigs.

The centrifugation of the blood, or of serum suspensions, was usually effected by means of an ordinary small electric centrifuge, carrying either 2 or 4 tubes, each having a capacity of 15 c.c. The speed of this machine on actual testing was found to be about 3000 revolutions per minute. The centrifuge tubes were plugged and sterilized before use.

In addition to this centrifuge, a larger one made by Burmeister and Wain, of Copenhagen, was found to be very useful. This machine has a maximal speed of 8000 revolutions per minute, each 100 revolutions being announced by a bell. It carries 6 tubes, each of 50-c.c. capacity, but smaller tubes can also be used. In order to keep the fluids sterile when the machine was operating at full speed, it was necessary to construct special centrifuge tubes. The revolving drum, it may be added, is 35 cm. in diameter and weighs 14 kilos. By means of this high-speed centrifuge it was possible to remove suspended matter to an extent otherwise impossible.

The incubation of sera was usually effected in a Roux water bath provided with a metallic regulator. As a rule the sera were placed in small Erlenmeyer flasks (30 to 50 c.c. capacity) so as to form a thin layer, thus being insured good contact between liquid and suspended matter.

The injection into the guinea-pig was always made in the external jugular vein, the animal being held on a Latapie board. The animal was then immediately released and the effects noted. The last faintest nasal twitch was taken as the death point. Individual variation in the susceptibility of the guinea-pig is to be borne in mind; a negative result does not prove the absence of the poison. It will be shown later on (Table 45), that not infrequently 2 guinea-pigs of equal weight, injected within a few seconds of each other, with the same material and dose, may behave totally differently; one may die of acute anaphylactic shock, while the other may show practically no effect. This, of course, holds true when approximately 1 lethal dose is given.

In the trypanosome work the animals were usually infected by intraperitoneal injection. Unless otherwise stated, they were bled shortly before death, at which time the number of trypanosomes approximated 200 per field of the No. 7 objective (eye-piece No. 3 being used). On centrifugation of such blood, the trypanosomes separated as a thick white layer on top of the erythrocytes. After the removal of the serum with a drawn-out bulb pipet, the organisms were taken up in a drawn pipet and transferred to a tube containing from 8 to 10 c.c. of 0.85% salt solution. Thorough agitation and recentrifugation, the operation being repeated 3 times, gave serum-free organisms.

The trypanosomes of surra, caderas, and dourine, and T. lewisi were employed in the earlier part of the work to show that anaphylatoxin-production is common to all of these, but the major part of the study concerned T. brucei. The relatively feeble toxicity of the sera treated with the first four organisms merely shows the poorness of the methods as first employed. With rat serum and short incubation they would without doubt give the same toxicity as serum treated with T. brucei; that is, a lethal dose of from 0.25 to 0.5 c.c.

SURRA TRYPANOSOME (T. EVANSI)

The study of anaphylatoxin began in connection with the work on immunity against surra. On the assumption that an anaphylatoxin was probably formed in vivo in the blood of infected rats, especially just before death, the sera obtained from such source were tested for the presence of this poison. The blood of the infected rats was defibrinated as quickly as possible, pooled, and centrifugated at 3000 revolutions for 5 minutes. The serum was then decanted and, usually, instead of being injected at once, it was placed on ice for 4 hours; this in subservience to the prevailing opinion that the primary toxicity of normal sera is reduced to a minimum by being kept for some hours at a low temperature.

An unusual toxicity of the trypanosomal, as compared with normal, serum seemed to be suggestive of in-vivo production since the icing of such sera after the removal of the organisms did not result in a further increase. Neither was there any marked decrease in toxicity on icing, for the initial titer sometimes persisted for an indefinite period. Thus a pooled surra serum which was kept in the ice chest at about 5 C. for 171 days produced acute anaphylactic death in dose of 2 c.c. (Table 1, Guinea-pig 10).

The results of these tests with the serum of surra are given in Table 1. The serum used for Guinea-pigs 1 to 4 was from the pooled blood of 6 heavily infected rats (200 per field); that for No. 5 was from 3 rats, bled while dying; that injected into Guinea-pigs 6 to 8 came from 3 likewise heavily infected rats. The serum employed for Guinea-pig 9 came from a single heavily infected rat, and was injected after being kept in the room for 1 hour; Guinea-pig 10 also received the serum from a single rat, which showed an extremely heavy infection (300 per field); this serum it is to be noted was kept iced for 171 days.

TABLE 1

ANAPHYLATOXIN IN DEFIBRINATED BLOOD OF SURRA RAT, AND IN GUINEA-PIG SERUM (NO. 11)

Guinea-Pig		Serum*		Result
No.	Weight	c.c.	Previous Treatment	
1	350	3.0	Iced 4 hr.	3½′; typical shock and autopsy
2	205	5.0†	" " "	Very slight
3	350	2.0	" " "	Severe
4	250	2.0	" " "	2′; typical shock and autopsy
5	300	5.0	" 3 "	Slight
6	265	4.0	" 4 "	Moderate
7	250	1.5	" " "	6′; typical shock and autopsy
8	212	0.75	" " "	Slight
9	250	2.0	Kept at 20 C., 1 hr.	1′ 15″; very severe shock
10	250	2.0	Iced 171 days	2′ 15″; very severe shock
11	260	4.5 (guinea-pig)	Kept at 37 C., 3 hr.; iced 24 hr.	Slight

* All injections were made intravenously except that of Guinea-pig 2.

† The injection of 2 was made intraperitoneally; 45 minutes later a second injection of 2 c.c. were given, without any effect.

KEY TO TABLES

In this and the following tables, the expression "typical shock" implies the full syndrome noted in anaphylaxis, namely: peripheral irritation, dyspnea, spasms, convulsions, urination. Likewise, "typical autopsy" means the findings of specific anaphylactic death, namely: permanent maximal distension of lungs, heart beating, low blood pressure, blood fluid, no clot.

The nonfatal results are conveniently indicated in the following manner:
Very slight.—The only effect is a slight change in respiration.
Slight.—Respiration irregular, no dyspnea, few jerky spasms.
Moderate.—Respiration irregular, some dyspnea, few jerky spasms.
Fair.—Some dyspnea and marked spasms.
Good.—Some dyspnea and marked spasms with few slight convulsions.
Severe.—Spasms, convulsions, dyspnea, thrown momentarily.
Very severe.—Same but remains thrown for some time; no marked apnea.
Near-kill.—Same with prolonged suppression of respiration and apparently dead. The time of death is indicated in the tables.

These preliminary tests seemed to confirm the assumption that the poison was present during life in the blood of the infected rats, but subsequent work showed that most of the toxicity observed in this and similar series of tests was really due to in-vitro changes. Hence the results given in this and the following tables are to be interpreted in that sense. It is to be noted that the surra serum in one test (No. 7) was fatal in as small an amount as 1.5 c.c., which at the time was the smallest toxic dose for any anaphylatoxin. Experience and a better understanding of the reaction have made it possible to produce with T. brucei within 15 minutes a serum having a lethal dose of from 0.25 to 0.5 c.c. On the other hand, it will be seen that at times relatively large doses, up to 5 c.c. (Guinea-pigs 2, 5, and 6), may be given with little or no more effect than follows the injection of normal rat serum.

In view of the results obtained with the caderas trypanosomes it would seem that the surra serum is less toxic. This conclusion, however, is not justified, for subsequent work with T. brucei has shown that the toxicity depends on the temperature and the duration of contact of the trypanosomes with the defibrinated blood or serum, and even on the mode of defibrination. In the surra tests the infected rats were bled late at night, when the room temperature was relatively low as compared with that of the afternoon, when the caderas blood was obtained. As indicated in the foregoing more toxic sera can be obtained by keeping the defibrinated trypanosomal blood for a few minutes at 37 C.

Only one attempt was made to render normal guinea-pig serum toxic by bringing it into contact with the washed parasites. For this purpose the trypanosomes from 6 heavily infected rats, after being washed 4 times with salt solution, by centrifugation, were added to 4.5 c.c. of normal serum in a small flask. The mixture was placed at 37 C. for 3 hours, then iced for 24 hours, after which it was centrifugated and injected into Guinea-pig 11 (Table 1). The result was practically nil.

The outcome probably would have been different had the injection been made immediately after the incubation as in the case of like tests with the caderas trypanosome (Table 2) and T. lewisi (Table 5).

CADERAS TRYPANOSOME (T. EQUINUM)

This organism was found to produce anaphylatoxin in rat serum about as readily as did that of nagana. The mere keeping of the defibrinated blood at room temperature for about an hour was sufficient to give rise to considerable toxin, as will be seen from the following experiment: Five white rats inoculated with T. equinum were bled 8 days later, at which time they showed 200 or more trypanosomes per field. The blood was defibrinated, pooled, and centrifugated at 3000 revolutions for 5 minutes. The serum was pipetted off and placed on ice for 16 hours, after which it was tested with the results shown in Table 2. The interval from the start of bleeding to the icing of the serum was 1 hour.

TABLE 2

Anaphylatoxin in Defibrinated Blood of Caderas Rat (1 to 4), and in Guinea-Pig Serum (5 and 6)

Guinea-Pig		Serum* (c.c.)	Result
No.	Weight		
1	250	2.0	2′; typical shock and autopsy
2	230	1.0	3′; " " " "
3	250	0.5	7′30″; " " " "
4	240	0.25	Severe
5	250	4.5 (guinea-pig)	45′; at first severe convulsions; autopsy typical
6	240	4.8 (guinea-pig)	2′; typical shock and autopsy

* Intravenously injected.

It will be seen from Table 2 that even 0.5 c.c. of the serum produced an acutely fatal shock. The icing of the serum in this and other experiments is of no particular value, the essential factor in the poison-production being the temperature and time during which the trypanosomes act on the serum. These conditions were particularly established with T. brucei and probably hold true for the other organisms of this type.

The deposit of trypanosomes obtained after centrifugation in this experiment was washed with 8 c.c. of 0.85% salt solution and then iced over night. The next day the operation of washing with salt solution was repeated 3 times, after which the trypanosomes were added to a small flask containing 5 c.c. of fresh normal guinea-pig serum. The latter was obtained by centrifugation of blood drawn by heart-puncture.

The mixture of serum and trypanosomes was placed at 37 C. for 14 hours and then centrifugated. This serum was tested on a guinea-pig (No. 5). The trypanosomes were now added to a second portion of 5 c.c. of normal guinea-pig serum and incubated at 37 C. for only 4 hours. After centrifugation, the serum was tested with the result shown in Table 2, No. 6.

The minimal lethal dose of the sera used in Tests 5 and 6 was not determined for obvious reasons. Consequently it may be questioned as to how much of the effect observed was due to the primary toxicity of serum and how much to the anaphylatoxin formed by the trypanosomes. While in general it is true that normal guinea-pig serum in dose of 4 to 5 c.c. gives little or no effect, it is nevertheless a fact, which our subsequent experience established, that, at times, 4 c.c. and even 3 c.c. of normal serum may kill. It is wrong to assume that a normal serum such as that of the guinea-pig or rat is innocuous in the doses given. This and many other like experiments were made before it was realized that a normal serum was subject to variation, as is also the recipient. A sufficient amount of pooled serum to permit duplicate control tests as well as duplicate tests with the treated serum is really needed to obtain results which will be reasonably certain.

It may be deduced from these experiments that the caderas trypanosome produces anaphylatoxin in rat serum and probably also in that of the guinea-pig. It is also possible that a prolonged incubation of the serum trypanosome mixture may yield a less toxic serum.

DOURINE TRYPANOSOME (T. EQUIPERDUM)

Only a limited number of tests were made with the sera of animals infected with this organism, sufficient to fix the general fact that it like other trypanosomes gives rise to anaphylatoxin. No attempt was made to induce toxicity in normal serum by the addition of washed dourine parasites. These tests like the preceding were made early in the course of the investigation before the importance of the incubation at 37 C. was ascertained, and while we were still looking for evidence of the presence of poison during life. Consequently, the results given in Table 3 represent merely the toxicity developed in the serum during the process of defibrination and centrifugation, which were carried on at room temperature. The icing of the clear serum, as indicated before, was probably without any effect as regards increasing the toxicity.

Five rats, showing about 200 tryanosomes per field, furnished the material for Guinea-pigs 1 to 4. After defibrination the pooled blood was centrifugated and the clear serum iced for 4½ hours before injection. For Guinea-pig 5, the blood of only 1 rat was used (100 per field); the serum was kept at room temperature for 2 hours and then injected. For No. 6, a guinea-pig inoculated 30 days before and showing only a moderate number of trypanosomes (20 per field), was used. The result in this case was a moderate shock with a drop in temperature to 34 C.

TABLE 3

Anaphylatoxin in Defibrinated Blood of Dourine Rat and Guinea-Pig

Guinea-Pig		Serum*		Result
No.	Weight	c.c.	Previous Treatment	
1	240	1.0	Iced 4 hr.	Very slight
2	205	2.0	" " "	Moderate
3	210	2.0	" " "	"
4	230	3.0	" " "	2′; typical shock and autopsy
5	230	1.7	Kept at 20 C., 2 hr.	50′; " " " "
6	250	4.8 (g.-pig)	Iced 4 hr.	Moderate

* Intravenously injected.

The tests recorded in Table 2 show that serum obtained under the conditions of the experiment possesses but a moderate degree of toxicity. The difference between these results and those gotten with caderas serum (Table 2) is striking, tho the conditions were much alike. It does not, however, mean that there is a real distinction between the two organisms concerned, but rather serves to emphasize the importance of such factors as temperature and duration of contact of the trypanosomes with the serum.

LEWIS TRYPANOSOME (T. LEWISI)

After preliminary experiments had shown that the pathogenic trypanosomes (surra, caderas, dourine, nagana) produced anaphylatoxin in rat serum, and that some of these organisms when washed were capable of toxifying normal guinea-pig serum, it was desirable to ascertain whether the relatively harmless T. lewisi was able to give rise to the same poison. The first tests with this material were decidedly negative and it seemed as if the poison-production was limited to the pathogenic forms. Subsequently, however, this was found to be incorrect. Given the right conditions, this organism is able to produce poison just as well as the pathogenic T. brucei, a fact which will be seen on comparison of Tables 5 and 6. This certainly holds true for guinea-pig serum and seemingly should be equally true for that of the rat.

The first failures to produce the poison in rat blood (Table 4) are subject to several interpretations, such as that they were due to the small number of organisms present and perhaps to the fact that these parasites do not disintegrate as readily as other forms. The absence of incubation, at 37 C., is clearly a decisive factor. Noteworthy is the fact that relatively large doses of rat serum (6 c.c.) obtained under these conditions may be innocuous, but it must not be inferred that normal rat serum is always as harmless when given in such doses.

The serum used for Guinea-pig 1 (Table 4) was from the pooled blood of 4 rats (30 per field); that employed for No. 2 was from a pool given by 6 rats (75 per field); that for No. 3 was from 3 rats (100 per field); while that injected into Nos. 4 and 5 came from 3 rats (150 per field). In each case the pooled blood was centrifugated at 3000 revolutions for 5 minutes, after which the serum was iced for the time indicated.

TABLE 4

ANAPHYLATOXIN IN DEFIBRINATED BLOOD OF RAT INFECTED WITH T. LEWISI

Guinea-Pig		Serum*		Result
No.	Weight	c.c.	Previous Treatment	
1	235	6.0	Iced 4 hr.	Nil
2	250	6.0	“ “ “	“
3	250	4.8	“ “ “	“
4	260	5.2	“ 12 “	Severe
5	205	4.6	“ “ “	15′; typical shock and autopsy

* Intravenously injected.

That the toxifying power of T. lewisi is not inferior to that of T. brucei is clearly demonstrated in the following series of tests (Table 5) with normal guinea-pig serum. In this series the same mass of washed tryanosomes was used successively to induce anaphylatoxin-production in 14 different lots of such serum. The trypanosomes were obtained from the blood of 3 rats, the serum of which was used for Tests 4 and 5, recorded in the preceding table. After removal of the serum the organisms were washed 3 times with 0.85% salt solution, and centrifugated each time at 3000 revolutions for about 2 minutes.

The washed trypanosomes were then added to 5 c.c. of fresh normal guinea-pig serum, in a small flask, and placed at 37 C. for 4 hours. The mixture was then centrifugated, and the serum removed and tested

(No. 1). The organisms were then added to another portion of 4 or 5 c.c. of fresh serum, mixed, incubated, and centrifugated as before. This second serum was then injected (after being iced over night) into Guinea-pig 2. This operation was repeated until 14 consecutive tests had been made. The experiment was then discontinued. Seemingly, the operation could have been repeated, with like results, an indefinite number of times.

TABLE 5

SERIAL PRODUCTION OF ANAPHYLATOXIN IN GUINEA-PIG SERUM BY WASHED T. LEWISI

Guinea-Pig		Serum		Result
No.	Weight	c.c.*	Previous Treatment with T. lewisi	
1	225	4.5	4 hr. at 37 C.	1′30″; typical shock and autopsy
2	250	5.0	“ “ “ “ “ and 12 hr. on ice	Very slight
3	245	4.3	4 hr. at 37 C.	2′; typical shock and autopsy
4	225	5.0	“ “ “ “ “	3′; “ “ “ “
5	180	3.8	“ “ “ “ “	2′; “ “ “ “
6	200	3.8	“ “ “ “ “	3′; “ “ “ “
7	205	4.5	2 “ “ “ “	2′; “ “ “ “
8	180	3.0	2 “ “ “ “	2′; “ “ “ “
9	260	3.8	1 “ “ “ “	2′; “ “ , no autopsy
10	205	4.0	1 “ “ “ “	2′; “ “ and autopsy
11	250	4.0	½ “ “ “ “	1′30″; “ “ “ “
12	260	4.0	“ “ “ “ “	5′; “ “ “ “
13	250	4.0	“ “ “ “ “	5′; “ “ “ “
14	230	4.0	“ “ “ “ “	2′; “ “ , no autopsy

* Intravenously injected.

It is noteworthy that the same mass of Lewis trypanosomes can be used to produce anaphylatoxin in guinea-pig serum for a considerable number of times. The results correspond exactly to those obtained with T. brucei (Tables 6 and 7). Similar serial toxifications with B. pyocyaneus, B. typhosus, etc., were obtained by Dold and Aoki,[8] but in their case the reaction seemed to come to an end with about the 10th test. In the light of subsequent facts it is indeed very doubtful whether there is any real end to the inducing power of bacteria or of trypanosomes, other than the exhaustion of the energy necessary for the induction.

Another important fact shown in Table 5 is that contact of the trypanosomes with the serum at 37 C. for one-half hour is apparently just as effective as a contact of 4 hours. Without doubt a shorter time would have sufficed, for in a similar series of tests with T. brucei (Table 6) a period of 15 minutes was enough. It is clear therefore that at a suitable temperature the reaction is developed at considerable speed. In view of the action of distilled water on normal rat serum

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[8] Ztschr. f. Immunitätsf., 1913, 18, p. 207. Levy and Dold, ibid., 1913, 19, p. 306.

(Part IX), it is likely that the optimal temperature will be found at or below 45 C. A series of tests with trypanosomes and rat serum at, or near, this temperature should give a maximal speed with the lethal dose well within 1 c.c.

NAGANA TRYPANOSOME (T. BRUCEI)

T. brucei was selected for the major part of this study, largely because of the intense infection which it produced in white rats within 2 or 3 days after inoculation. Consequently, a large amount of trypanosomes could be readily obtained from the blood after defibrination and centrifugation. At first the method of testing for anaphylatoxin was the same as that used with the surra trypanosomes and the other organisms, but later the icing of the trypanosome mixtures was abandoned in favor of continuous incubation and periodic testing.

Guinea-Pig Serum. — For a relatively small part of the work, normal guinea-pig serum was employed. Altho this is the most common reagent for anaphylatoxin-production, it is by no means the best, and hence in the course of time it was replaced almost entirely by the more responsive rat serum.

A few early tests indicated that normal guinea-pig serum could be toxified by T. brucei derived from infected rats. Thus, the trypanosomes obtained by centrifugation of the blood of 6 rats, after being kept for 7 days, iced and in contact with the cell deposit, were added to 5 c.c. of normal guinea-pig serum, incubated for 4 hours at 37 C. and iced over night. After centrifugation this treated serum, injected into a 205-gm. guinea-pig, caused typical shock with death in 2 minutes. The same trypanosomes were then treated with fresh guinea-pig serum in exactly the same way, and this serum in dose of 5 c.c. was also fatal to a 260-gm. guinea-pig in 3 minutes, the symptoms and findings being typical. A control test made at this time with 5 c.c. of normal guinea-pig serum, incubated and iced just as in the test proper, produced no effect. After a lapse of 7 days, the same trypanosomes were again treated with guinea-pig serum, as before, but this time the serum in dose of 5 c.c. produced only a severe shock. In these tests the same organisms apparently toxified the normal serum 3 times in succession. It might be objected that the effects observed, in spite of the one control test, might be due to a primary toxicity of the serum, since the dose employed was rather large.

To exclude such error it was necessary to inject smaller doses, and this condition was gradually met as seen from the following tables.

For the experiments recorded in Table 6 the blood of 4 rats, inoculated 3 days before and rich in parasites (200 per field), was used. The organisms were drawn off, after centrifugation, and washed with 8 c.c. of salt solution, and this washing was repeated 3 times. After final centrifugation they were added to 5 c.c. of normal guinea-pig serum, in a small flask, and incubated in the Roux water bath at 37 C. for the time indicated in the table. The clear serum obtained by centrifugation was then tested, while the trypanosomes were treated again with fresh serum. The experiment extended over 7 days and was then discontinued because it was deemed best to start a new series with a larger number of daily treatments. When not in actual use, the organisms were kept iced. There was no evidence of bacterial contamination. It will be seen from Table 6 that the toxifying power of the trypanosomes was maintained through 14 consecutive treatments with guinea-pig serum, the same as in the case of T. lewisi (Table 5). Even 15 minutes at 37 C. is sufficient to toxify the serum.

TABLE 6

I. SERIAL PRODUCTION OF ANAPHYLATOXIN IN GUINEA-PIG SERUM BY WASHED T. BRUCEI

Guinea-Pig		Serum		Result
No.	Weight	c.c.*	Previous Treatment with T. brucei	
1	250	4.5	2 hr. at 37 C.	Severe shock
2	260	5.0	" " " " "	3′; typical shock and autopsy
3	210	5.0	" " " " "	1′30″; " " " "
4	250	3.8	" " " " "	2′; " " " "
5	200	4.0	" " " " "	3′; " " " "
6	250	3.8	1 " " " "	2′; " " " "
7	200	3.8	" " " " "	4′; " " , no autopsy
8	260	4.5	½ " " " "	2′; " " and autopsy
9	260	3.0	¼ " " " "	Fair shock
10	300	4.0	½ " " " "	2′; typical shock
11	200	4.0	" " " " "	2′; " " and autopsy
12	230	4.5	¼ " " " "	3′; " "
13	230	4.5	" " " " "	3′; " "
14	205	3.3	50′ in room	4′; " " and autopsy

* Intravenously injected.

The trypanosomes for the next series were obtained from the blood of 4 rats inoculated 2 days before. The washing with salt solution and the further treatment were the same as described heretofore. The results are found in Table 7. In order to save animals no injections were made with Sera 10 to 13 and 15 to 19, but the results obtained with 14 and 20 show that the toxifying power of the organisms was kept up. The number of treatments, it will be seen, is double that reached by Dold and Aoki,[8] and it is probable that a great many more would have given like results. Altho this experiment extended over 8 days, the material showed no bacterial contamination.

TABLE 7

II. SERIAL PRODUCTION OF ANAPHYLATOXIN IN GUINEA-PIG SERUM BY WASHED T. BRUCEI

Guinea-Pig		Serum		Result
No.	Weight	c.c.*	Previous Treatment with T. brucei	
1	200	3.5	½ hr. at 37 C.	Severe shock
2	210	3.5	" " " " "	3′
3	215	3.0	" " " " "	2′
4	230	"	" " " " "	4′
5	250	"	" " " " "	Slight shock
6	250	"	" " " " "	Very slight
7	240	"	4 " " " "	4′
8	220	"	12 hr. at room temperature	3′
9	220	"	2 hr. at 37 C.	3′
				No tests made of Treated-sera 10 to 13 inclusive
14	230	"	" " " " "	2′
				No tests made of Treated-sera 15 to 19 inclusive
20	170	"	" " " " "	3′30″

* Intravenously injected.

Occasional failure to kill, as in Guinea-pigs 1, 5, and 6, must be expected, since the conditions are never exactly alike. Guinea-pigs do show individual resistance to anaphylatoxin, and sera from animals of the same species have been found to vary. The question of an initial toxic wave is also to be considered. When the same pooled serum is used for all the tests of a series, as in Table 8, the cause of failure is reduced either to individual variation on the part of the guinea-pig or to a change in the toxic state. If the amount employed represents more than 1 lethal dose, obviously the failures will be less in number (see Table 17).

TABLE 8

TOXICITY OF GUINEA-PIG SERUM TREATED WITH WASHED T. BRUCEI AND TESTED AT INTERVALS

Guinea-Pig		Incubation of Treated Serum at 37 C. (hr.)	Result of Intravenous Injection of 3 c.c.
No.	Weight		
1	172	¼	Practically nil
2	175	½	Slight
3	205	¾	3′10″; typical shock
4	210	1	Slight
5	195	1¼	Very severe shock
6	187	1½	Very slight
7	178	1¾	Fair

For the experiment recorded in Table 8, the blood of 3 nagana rats, after defibrination, was pooled and centrifugated at 3000 revolutions for 10 minutes; the trypanosomes were then removed and washed once with salt solution. They were then added to 23 c.c. of fresh normal guinea-pig serum, and the mixture was placed in ice for 1 hour. It was then put at 37 C., and at every quarter hour a portion was removed, centrifugated, and injected. It will be noted that a fatal dose of poison apparently was not formed until after incubation for 45 minutes, whereas in the tests recorded in Tables 6 and 7 this occurred at times in 30 and even in 15 minutes. This difference may find an explanation in the relative amounts of organisms and sera used in these experiments.

An apparent oscillation in the toxicity is indicated in the results as tabulated, and this feature is brought out more clearly in Chart 1. This and similar charts must be interpreted as expressing merely the results of the experiment, for if the injections had been made in duplicate at each period some variation undoubtedly would have been found. In other words, the curve indicates not so much a variation in the amount of the poison as a variation in the resistance of the animals.

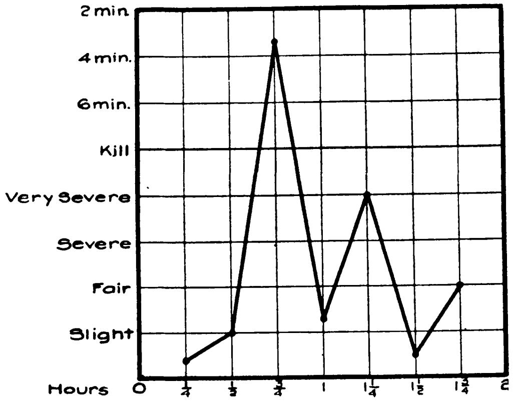

Chart 1. Apparent variation in toxicity of guinea-pig serum tested with T. brucei (Table 8); for meaning of terms, see Table 1.

Rat Blood and Serum.—In the early tests with infected rat blood, this was defibrinated at room temperature, pooled and centrifugated, the serum removed and iced for 4 hours, and then its toxicity tested. The great influence of time and temperature was not recognized until later. The first object here, as with the other trypanosomes, was to ascertain whether this organism gives rise to anaphylatoxin within the body of the rat. As seen from Table 9, which includes such early tests, it was readily shown that anaphylatoxin is produced in nagana rat blood, but whether all or a part or none of this exists in vivo remained to be determined. Further work showed that probably all of the results given in this table were due to the poison which is formed in vitro. A more certain and delicate method of detecting the presence of poison within the body was elaborated in time and will be taken up later on.

Table 9 includes the results obtained with 7 pools of nagana blood, the serum from each of which was treated as indicated. Pool 6 was obtained from 5 rats which were bled within an hour of death and consequently were extremely rich in organisms. On the other hand, Pool 4 was derived from the blood of 2 rats only moderately rich in parasites (50 per field). Pools 1 and 2 were also each obtained from 2 rats, but these were very rich in parasites (200 per field); Pools 3 and 7 came each from 6 rats equally rich in trypanosomes. Pool 5 was derived from 3 rats which were bled on the day following inoculation; at that time they showed only about 25 organisms per field.

TABLE 9

ANAPHYLATOXIN IN DEFIBRINATED BLOOD OF NAGANA RATS; SERUM KEPT AT 0 C. FOR 4 HOURS

Guinea-Pig		Serum		Result
No.	Weight	Pool	c.c.*	
1	215	1	2.0	4'; typical shock and autopsy
2	225	"	4.0	1'30"; " " " "
3	240	2	1.0	1'30"; " " " "
4	250	"	2.0	2'30"; " " " "
5	305	3	0.5	12'; " " " "
6	230	"	1.0	3'; " " " "
7	220	"	2.0	4'; " " " "
8	250	4	2.0	Nil
9	250	"	2.0	"
10	240	5	4.0	6'; typical shock and autopsy
11	240	6	0.25	Nil
12	250	"	0.50	Slight
13	240	"	1.0	"
14	250	"	4.0	4'; typical shock and autopsy
15	210	7	0.5	Slight
16	230	"	1.0	2'; typical shock and autopsy

* Intravenously injected.

Reference to Table 9 will show that the fatal dose ranged from 0.5 to 4 c.c. In view of the fact, which was recognized in time, that rat serum is very readily toxifiable, it is to be expected that in dose of 4 c.c. it would prove fatal. The extent to which anaphylatoxin is produced depends chiefly on the temperature and duration of operations preceding the transfer to ice. In all work with serum it must be remembered that the primary toxicity which is always present is merely increased by an external agent, in this case the trypanosome. Hence large doses of any treated serum are likely to give fatal results, since the toxicity of such serum represents the sum of primary and newly made anaphylatoxin. While it is possible to inject as much as 6 c.c. of rat serum without any effect, it is equally true that a slight variation in the method of defibrination may yield a serum which will kill in half that dose (see p. 506).

The influence of temperature on the formation of anaphylatoxin is clearly shown in Table 10. For these experiments the rats were bled on the second or third day of the infection, when the blood showed about 200 organisms per field. Each test was made with the blood of a single rat, except those the numbers of which are qualified as 8 and 8a, which indicates that they have a common blood source.

In the first two tests, the blood as soon as drawn was transferred to a centrifuge tube and immediately swung; the resulting serum was injected at once. It will be seen that such serum is practically without effect in the dose employed; that is, at this point no appreciable amount of poison is present.

It is possible that a much larger amount of such serum might have produced a toxic result, but it is self-evident that in the live, apparently well, tho heavily infected, animal the entire blood cannot carry much poison, otherwise a sudden and fatal intoxication would necessarily result. This may indeed be what happens in the terminal stages of the disease, especially in the guinea-pig, which is very susceptible to anaphylatoxin. The rat, however, which is insusceptible to enormous doses of anaphylatoxin, as will be later shown, may very easily carry such poison in its blood. As a matter of fact, transfusion experiments (Table 18) make it highly probable that some anaphylatoxin is present in the blood of infected rats.

In Test 3, the blood was spontaneously clotted in a centrifuge tube and kept at 37 C. for 1 hour. This serum produced a fatal shock with typical findings. For Guinea-pig 4 the blood was defibrinated for 1 minute and at once centrifugated; this serum again had but slight effect. The same was true for Tests 6 and 7, in which the defibrinated blood was kept at 37 C. for 2½ minutes. On the other hand, the incubation of the defibrinated blood for 5 minutes or more yielded a serum which was toxic in dose of 1 c.c.

A better result than the one just mentioned was shown in Test 5, which was made 2 years after the others in this table, with the express purpose of demonstrating the rapid speed of production. In this case the blood of a nagana rat (150 per field) was defibrinated in a water bath at 38 C. for 2 minutes, after which it was centrifugated for 4 minutes and the serum injected. The total time from the start of the bleeding to the end of the injection was 10 minutes. Several tests made at this time with blood of nagana rats, which was defibrinated for 1 minute in a bath at 38 C. and then centrifugated either at once or after further incubation for 1 or 2 minutes, gave little or no result; the blood incubated for 3 minutes, however, gave a serum which killed in dose of 2 c.c. in 11 minutes 30 seconds. It would appear from this as if the duration of the defibrination at 38 C. — that is, the agitation — was a factor to be considered in the production of anaphylatoxin.

The foregoing experiments demonstrate that the anaphylatoxin-production in trypanosomal blood occurs in vitro and, under favorable conditions of temperature, is but a matter of a few minutes. Normal rat blood, defibrinated or whole, and incubated at 37 C. for ¼, 1, 3, 6, and 12 hours, produced no effect when injected in dose of 1 c.c. When incubated for a much longer time, normal rat serum may, however, become toxic in this dose.

TABLE 10

ANAPHYLATOXIN IN WHOLE DEFIBRINATED BLOOD OF NAGANA RATS

Guinea-Pig		Serum			Result
No.	Weight	c.c.*		Previous Incubation at 37 C.	
1	210	1.0	Undefibrinated	0	Nil
2	230	"		0	Very slight
3	220	"		1 hr.	5′; typical shock and autopsy
4	205	1.0	Defibrinated	1′	Slight
5	205	" †		2′	5′20″; typical shock
6	203	"		2′30″	Very slight
7	205	"		"	Fair shock
8	210	"		5′	3′
8a	200	0.5		"	Slight
9	250	1.0		15′	5′
10	190	0.5		1 hr.	4′
10a	205	0.25		"	Slight
11	195	1.0		"	3′50″
11a	200	0.5		"	Slight
12	200	"		2 hr.	4′20″
12a	190	0.25		"	Severe
13	220	1.0		4 hr.	3′30″
13a	180	0.5		2 hr.	Slight
14	200	1.0		6 hr.	3′30″
14a	205	0.5		"	Good shock
15	200	1.0		12 hr.	4′
15a	175	0.5		"	2 hr.
16	200	1.0		"	5′
16a	208	0.5		"	Severe

* Intravenously injected.
† Defibrinated at 38 C.

Since it had been shown that practically all of the anaphylatoxin developed in nagana rat blood was formed after the latter was drawn, it was next in order to demonstrate that washed T. brucei in contact with normal rat serum likewise gives rise to the poison. An early series of tests made with this object in view is recorded in Table 11. For a given test the blood of a highly infected rat was at once chilled in ice water, then defibrinated and centrifugated. After removal of the serum the trypanosome layer was taken up with salt solution and again centrifugated. The deposit was then washed thrice with salt solution and finally it was taken up with 2 c.c. of normal rat serum and this mixture was then incubated for the time indicated. The serum after final centrifugation was injected in the dose indicated.

It will be seen that such mixtures may become toxic within 1 hour and that even 0.5 c.c. of such treated serum may be fatal. Tests 4, 5, and 8 were made with separate mixtures; Nos. 1, 2, and 3 were made with one mixture, while for Nos. 6 and 7 another was used. For Tests 6 and 7 the mixture, after incubation, was iced for 12 hours, and this fact may in part explain the failure to kill. For Test 5 the trypanosomes were not fresh, but had been iced in contact with the erythrocyte deposit for 6 days.

Even more marked results than those here given were obtained when, after centrifugation of the infected blood, the deposit of trypanosomes was taken up with its supernatant serum. Such tests were frequently resorted to, as will be seen in the following pages. Especial reference, however, may be made to Table 17.

TABLE 11

Anaphylatoxin Produced in Normal Rat Serum by Washed T. Brucei

Guinea-Pig		Serum		Result
No.	Weight	c.c.*	Previous Treatment with T. brucei at 37 C.	
1	205	1.0	1 hr.	4′
2	215	0.5	"	3′30″
3	210	0.25	"	Severe shock
4	190	1.0	"	3′
5	220	"	"	Moderate shock
6	205	"	4 hr. and 12 hr. at 0 C.	Severe "
7	190	"	" " " " "	" "
8	200	"	12 hr.	3′30″

* Intravenously injected.

Effect of Cold. — It has been shown heretofore that a surra rat serum, kept in the ice box for 171 days, was toxic in dose of 2 c.c. This result led to a series of tests with nagana serum under like conditions. The results are given in Table 12, from which it will be seen that the toxicity was unimpaired for 24 days. The final test made with the last portion of the serum on the 35th day gave a severe shock, but did not kill a guinea-pig of 300 gm. weight.

For this experiment the blood of 6 nagana rats was pooled, centrifugated for 5 minutes, and the serum transferred to a plugged, but not otherwise sealed, test tube and kept iced.

Effect of Heat.—The question of the production of anaphylatoxin by heated trypanosomes and by inactivated sera will be considered later. At this point it is desired to indicate the effect of heat on the anaphylatoxin formed by T. brucei in rat serum. In common with the anaphylatoxin of other origin, this poison is apparently unaffected by exposure to 60 C. Thus, a nagana serum the minimal lethal dose of which was 1 c.c. was placed in a Roux water bath at 60 C. for 1 hour. One cubic centimeter of this heated serum produced typical shock and death in 2½ minutes in a guinea-pig weighing 220 gm. When it was added to an equal volume of normal rat serum and incubated for 1 hour at 37 C., there was no increase in toxicity; the inducing power, whether due to ferment or to residual trypanosomal matter, had been lost or impaired.

TABLE 12

PERSISTENCE OF ANAPHYLATOXIN IN ICED SERUM FROM NAGANA RATS

Guinea-Pig		Serum		Result
No.	Weight	c.c.*	Iced	
1	230	1.0	4 hr.	2′
2	210	0.5	" "	Slight
3	240	1.0	16 "	2′30″
4	230	0.5	20 "	Moderate
5	225	1.0	40 "	3′
6	250	"	3 days	3′30″
7	250	"	4 "	3′
8	270	"	7 "	3′
9	230	"	14 "	3′
10	250	"	24 "	2′
11	300	"	35 "	Very severe

* Intravenously injected.

Since a serum on prolonged heating at 60 C. or at a higher temperature is soon gelatinized, it is not so easy to determine the action of such. If the serum can be rendered noncoagulable, without interfering with the test proper, then the action at these higher temperatures can be ascertained. This can be done by diluting the serum with several volumes of water, a feasible procedure in the case of rat anaphylatoxin having a lethal dose of 0.5 c.c. or less. A better method is one which has been in use in this laboratory for many years; this is based on the fact that a serum when dialyzed in a collodium sac at 50 C. for 5 minutes or more is noncoagulable even upon boiling. The increase in volume depends on the thickness of the sac and on the duration of the dialysis. Thus, in one test 1 c.c. of toxic serum representing a fatal dose was dialyzed, first at 50 C. for 15 minutes, then at 70 C., for

30 minutes. The total bulk, amounting to 2.25 c.c., injected into a guinea-pig (220 gm.) produced no effect.

This experiment goes to show that at 70 C., in half an hour, at least 1 lethal dose is destroyed. In Part IV, under the heading "Heating of the Mixture to 60 C. before Incubation," will be found tests on the agar anaphylatoxin from rat serum which show that at 70 C. for half an hour the poison is but partially destroyed. As to the identity of the anaphylatoxins prepared with trypanosomes and with agar there can be no doubt.

Attempts at Detoxification.—In line with previous observations of others, it will be shown later that freshly drawn blood becomes intensely toxic in the precoagulation stage and that after clotting occurs there is a marked decrease in toxicity. Normal sera, however, remain poisonous tho the toxicity of a given specimen may apparently vary, especially when judged by single animal tests. Since the sudden toxic wave inaugurated by the coagulation changes rapidly recedes, it was thought that the addition of normal blood, immediately after being drawn and before coagulation started, to anaphylatoxin might effect a reversal or destruction of the latter.

Several attempts were made in this direction by adding varying amounts of fresh guinea-pig blood (1 to 6 c.c.), obtained by heart-puncture, to a single lethal dose (1 c.c.) of toxic rat serum. After clotting, the mixture was allowed to stand for 1 hour, either at room temperature or at 37 C.; it was then centrifugated and the resulting serum injected into guinea-pigs. In each test, typical shock and death occurred in 3 minutes. Similar tests made by adding varying amounts of normal rat serum (1 to 3 c.c.) to the lethal dose of toxic serum (1 c.c.) likewise resulted fatally. It would appear therefore that neutralization of the poison does not occur under these conditions. A repetition of these tests, with a more toxic serum and without the incubation, may be desirable in view of the fact that the toxicity of organ extracts is influenced by normal sera. The toxicity of the extracts, however, is not to be considered as due to preformed poison, but to the anaphylatoxin to which they give rise in vivo on injection.

Several attempts were made to detoxify rat serum by adding a small amount of guinea-pig bile. In 2 of 5 trials, the mixtures apparently became nontoxic. However, unless such tests are made in duplicate, side by side, to exclude individual variability of the guinea-pigs, they can have but little value (see Table 45). Thus, in one test 0.3 c.c. of bile was added to a fatal dose of rat serum (1 c.c.) and, after standing 7 minutes, the mixture was injected, with only a very slight dyspnea resulting, but the test repeated at once with the same bile and serum caused death in 4 minutes. Two other tests gave similar results. The addition of cholesterin to toxic serum gave equally indefinite results.

Attempted Demonstration of Ferment Action.—Since it had been found that trypanosomes in contact with normal rat serum readily produced a very active anaphylatoxin, it was pertinent to inquire into the mechanism involved. It was quite natural to look on the process as enzymatic. The fact that heated trypanosomes, like heated proteins, starch, agar, etc., induce the same reaction, excluded the possibility of such ferment being derived from the organisms. The hypothetical ferment, therefore, must have its source in the normal serum, and, if it is brought into being by the presence of the trypanosomes, then it seemed reasonable to expect that a toxified serum should be capable of producing the same poison when diluted with one or more volumes of normal serum. When tests were made with this object in view, they apparently were successful and pointed to the existence of a ferment. Even a casual inspection of Tables 13 and 14 will reveal a suggestive resemblance to ferment action.

TABLE 13

APPARENT FERMENT ACTION: DILUTION OF TOXIC SERUM WITH NORMAL RAT SERUM (1 + 1 SERIES)

Exper.	Guinea-Pig		Serum		Result of Injection of 1 c.c. of Test Dilution
	No.	Weight	Dilution	Incubation at 37 C.* (hr.)	
A	1	205	1:2	1	2′
	2	250	1:4	1	3′50″
	3	230	1:8	1	Slight
B	1	240	1:2	1	3′
	2	230	1:4	2	Severe
	3	250	1:8	4	2′
	4	215	1:16	4	7′
	5	218	1:32	8	Severe
	6	220	1:64	8	"
	7	250	1:128	16	6′
	8	230	1:256	24	Severe
	9	200	1:512	36	Very slight

* This indicates the time which elapsed between the consecutive tests; that for No. 1 of each set is from the start of incubation.

Table 13 consists of 2 distinct experiments A and B. For the first the blood of 10 nagana rats was defibrinated, pooled, and centrifugated at 3000 revolutions. The toxic serum was kept in a capped tube on ice for 8 days before use. A preliminary test showed that 1 c.c. was fatal, whereas 0.75 and 0.5 c.c. produced only mild effects. Accordingly, 1 c.c. of this serum was added to 1 c.c. of normal rat serum, and this mixture, No. 1, was then placed at 37 C. for 1 hour; then 1 c.c. was taken out and tested. To the remaining half of Mixture 1, an equal volume of normal serum was added and this mixture, No. 2, was incubated and tested as before. In the same manner Mixture 3 was made and tested. It will be seen that Tests 1 and 2 resulted fatally; the latter representing a 1:4 dilution had but 0.25 c.c. of the original toxic serum.

Inasmuch as the third dilution failed to kill and the explanation seemed to be that not enough time had been allowed for the supposed ferment to act, the experiment was repeated, the digestion period being progressively increased. A portion of the same toxic serum used to start Exper. A was employed for Exper. B; the dilutions and tests were made consecutively as before. The results of this experiment confirmed and extended those obtained in the first trial. It will be seen from the table that even the seventh dilution of which the amount injected contained but $\frac{1}{128}$ c.c. of the original toxic serum, was fatal.

TABLE 14

Apparent Ferment Action: Dilution of Toxic Serum with Normal Rat Serum (1 + 3 Series)

Exper.	Guinea-Pig		Serum		Result of Injection of 1 c.c. of Test Dilution
	No.	Weight	Dilution	Incubation at 37 C. (hr.)	
1B	1	200	1:4	4	4′
2B	2	188	"	2½	Very severe
3B	3	200	"	2	3′30″
3C	4	200	1:16	8	4′30″
3D	5	205	1:64	8½	3′10″
4B	6	175	1:4	3	3′10″
5B	7	230	"	3	4′15″
6B	8	175	"	2¾	3′ 5″
7B	9	180	"	10	Moderate

The experiments of Table 13 were sufficiently suggestive to warrant further tests. These were made at different times during the following year, with a modified procedure. The toxic serum (1 c.c.) was diluted with 3 c.c. of pooled normal rat serum, giving a 1:4 dilution, usually designated as B. One cubic centimeter of B was diluted in like manner giving a 1:16 dilution, or C, and this process was continued to dilution D and even E. The advantage of this procedure is that the dilutions are made with the same pooled serum and that the mixtures are all incubated at the same time. Table 14 contains the results of tests with several different B dilutions; in the case of Experiment 3, the dilutions B, C, and D were tested. In the last 4 experiments the toxic sera were each diluted with inactivated rat serum; for Tests 6 and 7 the serum was heated at 56 C. for one-half hour, while for 8 and 9 the sera were placed at 60 C. for the same time.

The results given in Table 13 and for the first 5 tests of Table 14 could be easily interpreted as showing the presence of a ferment. Thus, in Table 13, 1 c.c. of a 1: 128 dilution killed, and 1 c.c. of the 1:256 dilution produced a severe shock; similarly, in Table 14 the 1: 64 dilution was fatal. These results are, however, extremely fallacious and misleading, having an entirely different significance. In the first place normal rat serum on incubation may acquire a marked toxicity, and further this inherent change is accelerated by the presence of minute amounts of trypanosomes. Altho the toxic sera were centrifugated at 3000 revolutions, such minute quantities were not excluded. In similar work with agar even more striking results were obtained, as will be seen on reference to Part IV.

The last 4 tests in Table 14 have a direct bearing on this point, since different treated sera were diluted each with inactivated serum. The same toxic serum used in Test 2 was diluted with serum heated at 60 C. and used for Test 8. Another toxic serum was treated with 60 C. serum for Test 9. For Test 7 the washed trypanosomes were added to 3 c.c. of 56 C. serum and the mixture iced for 1 hour, then centrifugated; the serum thus treated was then diluted with 3 parts of the same inactivated serum and this on incubation became toxic. Test 6 was made at the same time, but in this case the organisms were mixed with normal serum, and after icing and centrifugation this treated serum was likewise diluted with the 56 C. serum. This dilution it will be seen was also toxic.

It would appear from these tests that inactivated serum is toxified about as readily as normal serum. The small number of tests, however, renders it unwise to place too much stress on these experiments. Indications have been obtained showing that agar may toxify inactivated serum (Part IV). In general, it is held that anaphylatoxin cannot be produced in heated sera, the only exception being the observations of Seitz[9] and of Friedemann and Herzfeld,[10] but in these instances the shock produced was not acutely fatal, death occurring after 1 or more hours. It may be mentioned in this connection that trypanosomes which have been heated to 60 C. for half an hour, when added to normal rat serum and incubated, readily yield a toxic serum which in dose of 1 c.c. causes acute death.

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[9] Ztschr. f. Immunitätsf., 1912, 14, p. 91.

[10] Centralbl. f. Bakteriol., R., 1912, 54, Beiheft, p. 250.

It will be seen from what has been said that the activity of a ferment as regards anaphylatoxin-production is not established by these tests. The presence and action of such an agent are rendered still more doubtful by experiments made with sera centrifugated at very high speed (8000 revolutions).

Comparative Effect of High- and Low-Speed Centrifugation.—The work on agar anaphylatoxin, to be described in Part IV, first revealed the important fact that a treated serum apparently possessing a catalytic or ferment action, completely loses that property if centrifugated at a speed of 8000 revolutions for from 20 to 40 minutes. It seemed therefore that the catalytic action is not due to a substance in solution, but rather to something in suspension which is not fully removed by short centrifugation at low speed. To maintain the ferment idea it must be assumed that it is held loosely absorbed by the fine suspensoid.

In view of the results obtained with agar-treated serum it was desirable to see if this held true for the trypanosome serum. Four series of tests were made in which the comparative effect of centrifugation at 3000 and at 8000 revolutions was studied. The results were essentially identical with those obtained in the work with agar. It will be sufficient to describe 2 of these experiments.

For the tests given in Table 15, 7 heavily infected nagana rats were bled. In each case the heart pipet was instantly sealed with vaselin, plunged into ice water and the blood defibrinated. The operation of drawing and defibrinating the 7 bloods took 20 minutes. The bloods were then pooled in an iced flask and divided into 2 portions of 12 c.c. each, which were then placed in previously iced centrifuge tubes.

One portion was then centrifugated at 3000 revolutions for 7 minutes, at the end of which the corpuscles and the bulk of trypanosomes were thrown down. The supernatant serum was cloudy, however, and a rapid examination showed that this was due to the presence of organisms. The toxicity of 1 c.c. of this serum was tested at once, the balance being placed at 37 C. in the Roux water bath, and then tested at the intervals noted.

The centrifugation of the second portion, at 8000 revolutions for 40 minutes, was started at the same time as that of the first portion.

The water-clear serum thus obtained was tested at once, and the balance, placed at 37 C., was tested at the times shown in the table.

TABLE 15

I. COMPARISON OF EFFECTS OF DIFFERENT CENTRIFUGATION-RATES ON ANAPHYLATOXIN-PRODUCTION IN DEFIBRINATED BLOOD OF NAGANA RAT

Guinea-Pig		Serum			Result
No.	Weight	Centrifugation	Incubation at 37 C. (min.)	Intravenous Injection (c.c.)	
1	190	3000 r. p. m. for 7 min.	0	1.0	Nil
2	185		15	0.5	3′20″
3	185		19	0.25	3′45″
4	179		66	0.125	Severe
5	200	8000 r. p. m. for 40 min.	0	1.0	Very slight
6	203		15	"	Nil
7	205		30	"	Very slight
8	202		55	"	Nil

In this experiment special care was taken to check or prevent any action of the trypanosomes previous to the centrifugation. Hence, the precautions to keep the material cold. The time which elapsed from the beginning of the first bleeding to the injection of Guinea-pig 1 was 38 minutes; that to injection of Guinea-pig 5 was 86 minutes.

It will be seen from Table 15 that the cloudy serum, which was not toxic in dose of 1 c.c. when tested at once, on incubation rapidly became toxic. The lethal dose of 0.25 c.c. shows that the same degree of toxicity can be obtained with these organisms as with agar. By contrast, the clear serum was practically without effect when tested at once, or after incubation. The occasional twitches or slight jerky spasms were such as are often obtained with like amounts of normal serum (autoanaphylatoxin).

This experiment clearly shows that some trypanosomes must remain in the serum in order that it may become toxic. Their rapid and complete removal leaves the serum practically in a normal state.

The failure to kill with the clear serum cannot be explained by the supposition that the poison was either altered or thrown down by the prolonged high-speed centrifugation. Such a view would find no support in the next experiment (Table 16), and furthermore, it may be said that an actual test of a serum having a lethal dose of 0.25 c.c. showed that centrifugation at 8000 revolutions for 40 minutes was without effect on the toxicity.

As illustrative of the need of attention to details and speed, the following experiment is given (Table 16). It was made some days prior to the preceding one. Six infected rats were bled in 25 minutes; each heart pipet, after defibrination, was placed in ice water. The bloods were pooled and placed in cracked ice for 1 hour. The blood was then divided into 2 portions; one portion was centrifugated at 3000 revolutions for 4 minutes, while the other portion was swung at 8000 revolutions for 20 minutes. The first serum was very cloudy, because of the presence of some trypanosomes, while the second serum was perfectly clear. The time which elapsed from the first bleeding to the injection of No. 1 was 1 hour, 53 minutes; to injection of No. 5, 2 hours, 36 minutes.

TABLE 16

II. Comparison of Effects of Different Centrifugation-Rates on Anaphylatoxin-Production in Defibrinated Blood of Nagana Rat

Guinea-Pig		Serum			Result
No.	Weight	Centrifugation	Incubation at 37 C. (min.)	Intravenous Injection (c.c.)	
1	180	3000 r. p. m. for 4 min.	0	1.0	5′30″
2	200		3	0.5	3′40″
3	185		12	0.25	Very severe
4	215		38	"	3′45″
5	200	8000 r. p. m. for 20 min.	0	1.0	3′25″
6	203		8	0.5	Very slight
7	210		30	"	Moderate
8	205		60	"	Severe

To the fact that the blood of each rat was defibrinated before icing, and to the further fact that the pooled blood, tho iced, was kept for an hour, is due the toxicity seen in Nos. 1 and 5. This means that in the second series the trypanosomes were removed after they had given rise to some poison. Unless a soluble ferment was present one would not expect an increase in the toxicity of this serum. There is, however, an apparent increase shown in Test 8, and this has also been noted in corresponding experiments with agar. It is more than likely that this effect is due to the animal's having a greater susceptibility. The same explanation holds true for the apparent increased toxicity of the serum in No. 4 as compared with No. 3. When duplicate tests are made variations of this kind are frequently met with (Table 45).

Maximal Toxicity.—The foregoing tests having shown that it was possible to obtain an anaphylatoxin in about 15 minutes such that 0.25 c.c. was lethal, it was desirable to see whether this result could be obtained with reasonable frequency. With this object in view considerable effort was devoted to duplicating these results, especially that in Table 15, No. 3. The outcome of these tests, however, was most discouraging. Frequently, severe symptoms were obtained with 0.25 c.c. of the treated sera, but fatal results with that dose were extremely rare; in fact, only once, apart from the results tabulated, was this lethal dose obtained. In 12 of 24 tests it was possible to toxify the serum in 15 minutes at 37 C. so that 0.5 c.c. was fatal.

It is difficult to offer a satisfactory explanation of such failures, for without doubt several factors are concerned. The sera of animals unquestionably vary in the ease with which they can be toxified under apparently identical conditions. Furthermore, the proper amount of organisms or their state is a matter of moment just as it is in the case of agar. Again, as pointed out heretofore, single tests of toxicity are not a certain means of detecting the presence of a minimal lethal dose; a better indication is obtained when 2 guinea-pigs are injected at each period.

It is a very significant fact that the maximal toxicity is often reached within a few minutes. From the ferment standpoint one would expect a progressive increase, but such does not seem to be the case. Whether agar or trypanosomes are used as toxifying agents, the greatest toxicity, represented by a lethal dose of from 0.2 to 0.25 c.c., is frequently obtainable within 15 minutes or less, the incubation being at 37 C.

Apparent Loss of Toxicity.—In connection with the work on agar anaphylatoxin it was frequently found that the toxicity apparently rose and fell, thus indicating an oscillation or wave character. Similar tests made with trypanosome serum exhibited something of the same behavior. One of these experiments, made with guinea-pig serum, has already been described (Table 8, Chart 1); another test was made with rat serum. In view of the greater ease with which this serum can be toxified, it was possible to employ a much smaller dose (0.5 c.c.). The results are consequently the more instructive, as will be seen from Table 17 and Chart 2.

For this experiment, 6 heavily infected rats were bled in 20 minutes; the defibrinated pooled blood was at once centrifugated at 3000 revolutions. The trypanosome layer with the supernatant serum was then pipetted off, mixed, and placed in cracked ice for 1 hour. The only purpose of the latter step was the convenience of starting the tests at that time. It is possible that icing has an unfavorable action on the toxicating power of the organisms. The mixture was then placed at 37 C.; and at each quarter-hour period 1 c.c. was removed, centrifugated, and 0.5 c.c. of the clear serum injected. As soon as the first fatal shock was obtained (No. 4), the entire mixture was centrifugated at 3000 revolutions for 8 minutes and the resulting serum was then returned to the water bath. It follows therefore that the tests, beginning with No. 5, were made with serum from which the organisms had been removed; that is to say, the oscillations from this point on occur in a practically cell-free serum. Seemingly, the reaction when once initiated by the alien matter, in this case trypanosomes, continues even tho the original inciting cause be removed.

TABLE 17

Apparent Variation in Toxicity of Rat Serum Treated with T. Brucei

Guinea-Pig		Incubation of Serum at 37 C. (hr.)	Result of Intravenous Injection of 0.5 c.c.
No.	Weight		
1	182	¼	Severe
2	183	½	Very severe
3	179	¾	" "
4	200	1	3′25″
5	205	1¼	Good
6	195	1½	3′50″
7	181	1¾	3′55″
8	197	2	Slight
9	175	2¼	3′50″
10	175	2½	5′
11	174	2¾	7′ 5″
12	179	3	3′45″
13	195	3¼	8′20″
14	181	3½	Slight
15	176	3¾	Very slight
16	173	4	Fair
17	171	4¼	4′20″

The greater frequency of fatal results in this series as contrasted with that in which guinea-pig serum was used (Table 8) must be ascribed to a greater toxicity of the treated rat serum. The guinea-pig serum barely contained 1 lethal dose, whereas the rat serum probably contained nearly 2 lethal doses, since it has been shown that it can develop a titer of 0.25 c.c. The occasional failure to kill, or even to produce a severe shock, means not so much that the poison has decreased but rather that the resistance of the animal has increased (see Table 45). This resistance obviously becomes less marked when the serum injected contains several lethal doses. One thing which is perfectly clear is that the poison once made persists in the serum at 37 C. for at least 4 hours.

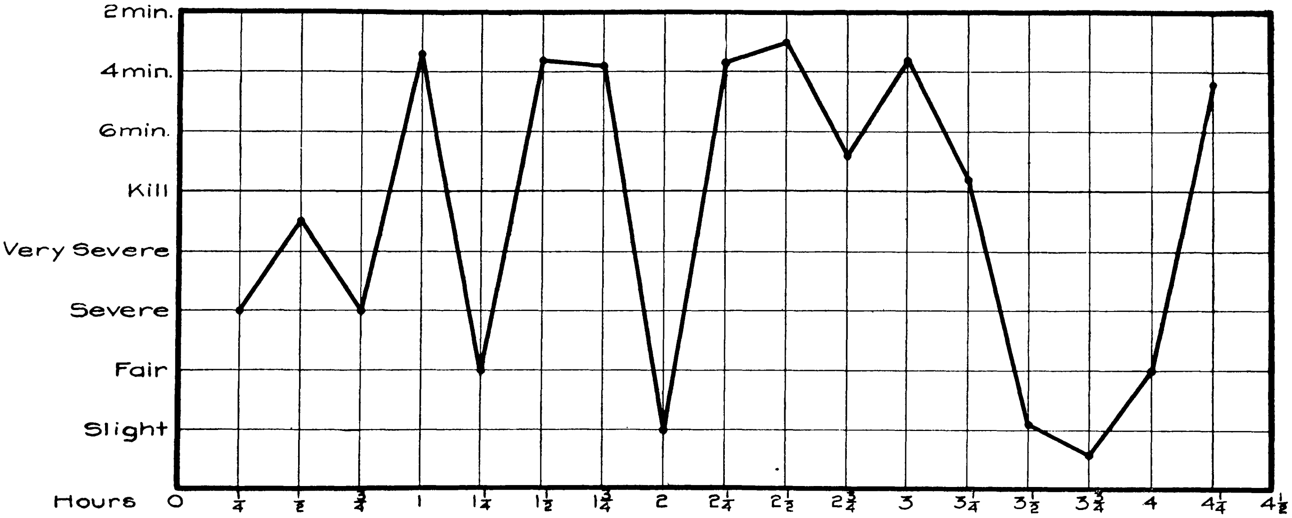

Chart 2. Apparent variation in toxicity of rat serum treated with T. brucei (Table 17).

Anaphylatoxin in Infected Rats.—Somewhat paradoxical is the fact that trypanosomes when incubated with normal serum readily yield a fatal poison, whereas the same organism in contact with the plasma of the living animal apparently does not do so. In the case of the very susceptible guinea-pig a very small amount of such poison if formed within the body ought to kill. With the rat the condition is otherwise, since it is extremely resistant to the poison. The phenomenon is not particularly difficult to understand from the standpoint of a physical reaction. It parallels perhaps the incoagulability of blood during life. The probable explanation is that while the organisms tend to change the physical state of certain plasma colloids, the enormous mass of other colloids in the plasma and in the cells exerts a check on the extent of this change. Certain it is, that when the infected blood is drawn into a test tube, the inhibitory action of the body mass is absent and hence the trypanosomes rapidly induce this change; in the absence of organisms the drawn blood passes through a similar reaction which finds its expression in the production of precoagulation toxicity (auto-anaphylatoxin), and in fibrin coagulation. While the organisms multiplying within the blood, or other parts of the body, are unable to produce an acute anaphylactic shock, they may bring about a gradual and moderate change represented perhaps by chronic intoxication and cachexia, seen especially in rabbits.

The studies on agar anaphylatoxin showed that the rat possesses a remarkable resistance to this poison. A dose sufficient to kill 100 guinea-pigs can be tolerated by the rat. This fact was first utilized to demonstrate the in-vivo production of anaphylatoxin in agar-shocked rats, and in the shock of specific anaphylaxis. It was eventually applied in determining whether or not this poison was present in the blood of heavily infected nagana rats. As a consequence of this natural resistance, such infected rats may well carry sufficient poison in their blood to kill several guinea-pigs. The rapid transfusion of a suitable amount of blood ought to establish the presence or absence of the poison.

For the following experiment a number of large rats, weighing about 180 gm., were inoculated with T. brucei. When the number of parasites in the blood had increased to about 100-150 per field, they were used for the tests. A syringe needle was introduced into the exposed heart, the desired amount of blood was drawn up and at once injected into the jugular vein of a guinea-pig.

It is essential for the success of this experiment that the transfer time shall be as short as possible. A delay of a few seconds, especially with a large amount of blood, may result in a fatal shock even with normal blood, through the development of precoagulation anaphylatoxin. In Table 66, Part VI, will be found the results of transfusions of normal rat blood into guinea-pigs and these may serve as controls for this series. It will be seen that 4 c.c. of normal rat blood can be transfused with little or no effect, provided the transfer time is kept within about 45 seconds. Reference to Table 18 will show that this amount of nagana blood, with like transfer time, is fatal. This result would be more convincing if the transfer time was reduced to 20 or 30 seconds, a speed which is only possible with very large rats. It certainly would be of interest to repeat these tests with very large, almost moribund rats in which the maximal change could be expected.

As the tests stand, it may be conceded that a portion of the anaphylatoxin demonstrated by this method is made in vivo; a second portion by the trypanosome action within the syringe, and a third portion by precoagulation changes in the blood itself, likewise in the syringe. The sum of these three factors is expressed in the observed effects. It would not be logical to assume that all the anaphylatoxin present in the injected blood was formed within the rat.

TABLE 18

THE IN-VIVO PRODUCTION OF ANAPHYLATOXIN IN RATS INFECTED WITH T. BRUCEI

Guinea-Pig		Intravenous Injection (c.c.)	Transfer Time* (sec.)	Result
No.	Weight			
1	180	2	30	Practically nil
2	183	"	33	Nil
3	190	3	37	Slight
4	215	4	51	3′ 4″
5	215	"	40	4′14″

* By "transfer time" is meant the interval from the entrance of the syringe into the heart of the rat until its withdrawal from the vein of the guinea-pig (see Table 65).

Intravenous Injection of Washed Trypanosomes. — It has been pointed out that the trypanosomes in a heavily infected rat may give rise in vivo to some anaphylatoxin, but that in the case of the guinea-pig this must of necessity be less than 1 lethal dose, since otherwise acute death would follow. The mass of the body colloids must be looked upon as exerting a reversive action pari passu with the multiplication of the organisms, but in drawn blood this factor is removed and anaphylatoxin is rapidly formed. The sudden injection of a very large amount of trypanosomes might very well result in a disturbance of equilibrium sufficient to bring on a typical anaphylactic shock. It is known that injections of bacteria, kaolin, and silicic acid can produce an acute fatal shock, and, as will be shown, the same is true for agar.

Six attempts were made to shock by this method. The washed organisms from 4 to 6 nagana rats were suspended in 1 c.c. of salt solution and injected into a guinea-pig of 200 gm. weight. In one such test marked prostration followed, the temperature in 20 minutes dropped to 34 C., and in 45 minutes it was below 29 C. Marked bleeding was present, indicating a noncoagulable blood; spasm appeared in about an hour and death occurred in 1¼ hours. In another test jerky spasms appeared in 3 minutes and continued for some time; the temperature in 20 minutes dropped to 35.5 C., but recovery took place. In the other tests no appreciable effect was observed.

Since subsequent experiments with injections of agar demonstrated the importance of a suitable dilution, it is highly probable that better results would have been obtained had the organisms been suspended in about 10 c.c. of salt solution or distilled water. The two results mentioned indicate that a 'trypanotoxin' action can be induced by the intravenous injection of washed trypanosomes into a guinea-pig. This action, as with so-called 'endotoxins' in general, does not mean that the cells are poisonous in the common acceptance of the term; they merely induce in vivo the same reaction as in vitro. This interpretation of endotoxin action will be made clear in connection with the agar work (Part VI).

Injection of Normal Sera into Nagana Guinea-Pigs.—Since trypanosomes react promptly with normal sera in vitro giving rise to anaphylatoxin, it was of interest to learn whether the injection of a normal serum into a heavily infected guinea-pig would bring on a similar reaction. Inasmuch as the guinea-pig serum does not give rise to a very active poison outside of the body it was hardly to be expected that it would do otherwise when injected into the infected animal. But with rat serum the result might well be different because of its capacity to yield a poison the lethal dose of which may be 0.25 c.c. or even less. With this object in view several tests were made with homologous serum and with the heterologous rat serum.

The guinea-pigs were tested on the 5th to the 10th day after inoculation; at that time they showed from 25 to 100 trypanosomes per field. The guinea-pig serum was injected in a dose of 5 c.c. No immediate effect was noted. In one animal convulsions were observed in 2 hours and death occurred 6 hours after the injection. The fact that an untreated infected control died about the same time made it probable that the effect observed was not due to the injection. It should be added that this amount of guinea-pig serum may itself at times be fatally toxic.

In several of the treated animals there was, apparently, a few hours after the injection, an enormous increase in the number of parasites, and it seemed as if this was due to the serum injected. If this observation were true, it would be of value as a means of obtaining large amounts of parasites. Unfortunately, a number of attempts made to confirm this apparent multiplication were without result. The increase noted was merely such as is occasionally met with in the guinea-pig prior to death. It may be added that a somewhat similar observation on infected rabbits and guinea-pigs, subjected to anaphylactic shock, was reported by Scott.[11]

Toxicity of Normal Rat Serum.—The injection of rat serum into infected guinea-pigs gave more promising results. Thus, while in the first trial, 5.3 c.c. of normal rat serum were injected with no noticeable effect other than a rapid increase in the number of parasites, in a second test, 5 c.c. produced a typical shock and death in 2 minutes; while in a third, the same result was produced by 4 c.c.

These tests were made at a time when it was believed that normal rat serum was not toxic in dose of 6 or 7 c.c. The death could not be ascribed to specific anaphylaxis, because the guinea-pigs had been inoculated with a strain maintained in these animals. It seemed therefore that the object of the experiment had been realized, showing that the trypanosomes could react in vivo with the injected rat serum. In due course of time, however, it was found that normal rat serum could be toxic in the doses mentioned, and this fact made it probable that the observed deaths were due directly to the injected serum.

The experiment was therefore repeated on two different occasions. In one of these tests the infected guinea-pig received 4 c.c. without any effect. In a second test, an infected animal after receiving 4 c.c. of a pooled serum died of typical shock in 3½ minutes; but 2 controls which were given 3 and 4 c.c., respectively, of the same pooled serum likewise died in 3½ and 4½ minutes!

It is evident therefore that normal rat serum may, at times, cause typical anaphylactic shock and death in dose of 3 or 4 c.c. It may be added that this pooled serum was derived from 8 rats. The blood as fast as removed from one was discharged into a cylinder provided with a rod. The operation for the lot took half an hour. The combined blood was then thoroughly whipped, centrifugated, and the resultant serum iced for about 6 hours before use. The remarkable toxicity of this serum might have been due to the delayed mass defibrination; it might also have been due to some one of the rats having a chance infection with T. lewisi. Unfortunately, no examination was made to exclude the latter possibility. If the blood was from normal animals, then the fatal dose of 3 c.c. represents the smallest lethal dose of normal rat serum.

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[11] Bull. Soc. path. exot., 1911, 4, p. 674.

It may be concluded from the foregoing that the trypanosomes within the body of a guinea-pig cannot toxify an injected serum. Apparently, the condition (body mass) which restrains the production of anaphylatoxin in the plasma of the infected animal, also exerts a like control over an alien serum.

SUMMARY

Anaphylatoxin was produced by 5 different trypanosomes, including the nonpathogenic T. lewisi.

It is made not only by the living cells, but also by the dead, more or less autolyzed, cells, and even by such when heated to 60 C.

The same mass of trypanosomes can be used repeatedly to toxify different lots of serum without apparently any limit to their inducing power. This serial production in one experiment extending over 8 days, was carried through 20 tests with no indication that the organisms were weaker than at the beginning.

Rat serum is preferable to guinea-pig serum since it yields a poison which may be 12 or more times as active as that obtained with the latter; the respective lethal doses being 0.25 c.c. of the former and 3 of the latter.

The speed of poison-production under favorable conditions is very rapid and quickly reaches a maximum, the poison then persisting for a long time. Thus, infected defibrinated rat blood or serum, when incubated for 1 or 2 minutes, may become fatally toxic. With an incubation of about 15 minutes, it is possible to produce a toxic serum such that 0.25 c.c. will cause acute anaphylactic death.

Sera inactivated at 56 C. or 60 C. for half an hour apparently can be toxified.

The anaphylatoxin was found to persist at 37 C. for more than 4 hours; at about 0 C. it may persist for an indefinite time. Thus, a surra serum was still toxic after icing for 171 days; a nagana serum was likewise active for 35 days. Attempts at detoxifying by addition of normal serum, bile, or cholesterin resulted negatively.

Toxic sera can induce toxicity in normal sera, the mixtures representing a high dilution of the former. This result is not due to the action of a ferment, but to minute amounts of trypanosomes still present in the inciting serum; or, in the case of very prolonged incubation, to the production of autoanaphylatoxin.

A comparison of the effects of different centrifugation-rates on chilled, rapidly defibrinated blood shows that sera obtained at slow speed readily become toxic; the sera secured at a very high speed do not.

Transfusion experiments indicate the possible presence of anaphylatoxin in heavily infected rats. The formation of this poison in corpore may lead to sudden deaths or to chronic intoxication or cachexia.

The injection of normal rat serum into infected guinea-pigs does not result in the in-vivo production of poison; any effect observed is due to the primary toxicity or autoanaphylatoxin of such injected serum.

The toxicity of trypanosomal sera, when tested at regular intervals, appears to show more or less oscillation. Similar variations are met with in tests with anaphylatoxins produced by other agents, and even in normal sera. They are not due to changes in the amount of poison, but to varying resistance of the test animals.

The injection of large amounts of washed trypanosomes into guinea-pigs may result in the production of anaphylatoxin in corpore. The effects are not due directly to the organisms, but to the disturbance in the colloidal state of plasma constituents, caused by the alien material.

The 'trypanotoxins' are therefore disturbers of equilibrium; the result is a poison-production in vivo as well as in vitro. The mode of action of endotoxins in general will be found to be of the same nature.

The participation of a ferment in this reaction is contraindicated by the speed of production, the rapid attainment of maximal level, the behavior of inactivated sera, and by the results of centrifugation.

The syndrome of symptoms and autopsy findings consequent on the injection of the trypanosome anaphylatoxin are those of the intoxication of true anaphylaxis. The two poisonings are to be considered as identical.